Human T Cells Express a Functional Ionotropic Glutamate Receptor GluR3, and Glutamate by Itself Triggers Integrin-Mediated Adhesion to Laminin and Fibronectin and Chemotactic Migration[1]

Yonatan Ganor,[2]* Michal Besser,[2]*† Naomie Ben-Zakay,* Tamar Unger,* and Mia Levite[3]*†

T cells may encounter glutamate, the major excitatory neurotransmitter in the nervous system, when patrolling the brain and in glutamate-rich peripheral organs. Moreover, glutamate levels increase in the CNS in many pathological conditions in which T cells exert either beneficial or detrimental effects. We discovered that normal human T cells, human T leukemia cells, and mouse anti-myelin basic protein T cells express high levels of glutamate ion channel receptor (ionotropic) of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtype 3 (GluR3). The evidence for GluR3 on T cells includes GluR3-specific RT-PCR, Western blot, immunocytochemical staining and flow cytometry. Sequencing showed that the T cell-expressed GluR3 is identical with the brain GluR3. Glutamate (10 nM), in the absence of any additional molecule, triggered T cell function: integrin-mediated T cell adhesion to laminin and fibronectin, a function normally performed by activated T cells only. The effect of glutamate was mimicked by AMPA receptor-agonists and blocked specifically by the selective receptor-antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6-nitro-7-sulfamoylbenzo[*f*]quinoxalin-2,3-dione (NBQX), and by relevant anti-integrin mAbs. Glutamate also increased the CXCR4-mediated T cell chemotactic migration toward the key chemokine CXCL12/stromal cell-derived factor-1. GluR3 expression on normal, cancer and autoimmune-associated T cells and the ability of glutamate to directly activate T cell function could be of substantial scientific and clinical importance to normal neuroimmune dialogues and to CNS diseases and injury, and especially to: 1) T cell transmigration to the CNS and patrolling in the brain, 2) T cell-mediated multiple sclerosis, and 3) autoimmune epilepsy, as neurotoxic anti-GluR3 Abs are found and suspected to cause/potentiate seizures and neuropathology in several types of human epilepsies. Thus far, GluR3 was found only on neurons and glia cells; our results reveal a novel peripheral source of this antigenic receptor. ***The Journal of Immunology,*** **2003, 170: 4362–4372.**

Under physiological conditions, T cells frequently patrol the CNS. T cells are also present in the CNS in various brain pathologies where they either cause/augment the pathology (e.g., T cell-mediated encephalomyelitis as in multiple sclerosis (MS)[4]) (1,2) and in infection by Theiler virus (3) or combat it (e.g., T cell-mediated clearance from the brain of encephalomyelitis-inducing viruses or T cell-dependent neuroprotection after neuronal injury) (4–6). The factors responsible for regulating T cell activities within the brain and for allowing a direct cross-talk between T cells and resident neurons and glia cells (7) are still unknown, and their identification may have important physiological and clinical implications.

In recent years, we found that several neurotransmitters and neuropeptides, among them dopamine, gonadotrophin-releasing hormone (GnRH) I and II, somatostatin, substance P, calcitonin-gene-related peptide, and neuropeptide Y can by themselves, in physiological concentrations, interact directly with their cognate receptors expressed on the T cell surface and trigger T cell functions, among them cytokine secretion, integrin-mediated adhesion to extracellular matrix (ECM) glycoproteins, chemotactic migration and gene expression (8–12). Can this be also the case for L-glutamate, the major excitatory neurotransmitter in the nervous system, mediating most of the excitatory transactions between CNS neurons? T cells can be expected to encounter glutamate when routinely inspecting the brain, as well as in various glutamate-rich peripheral organs such as the liver, kidney, lung, muscle, and blood. In addition, there is a kaleidoscope of pathological conditions that display a neuroinflammatory component and in which glutamate levels increase substantially, causing neuronal death by a mechanism called excitotoxicity (13). These pathological conditions, in which T cells could possibly encounter glutamate, include traumatic brain injury, acute brain anoxia/ischemia (i.e., stroke), epilepsy, glaucoma, meningitis, brain neurodegeneration associated with different chronic diseases such as AIDS-associated dementia, MS, amyotrophic lateral sclerosis, and Alzheimer's disease (reviewed in Ref. 14).

Glutamate has two broad families of receptors: the ionotropic receptors that are glutamate-gated ion channels; and the metabotropic receptors coupled to G proteins. The ionotropic receptors are

*Weizmann Institute of Science, Rehovot, Israel; and †Sackler Faculty of Medicine, Tel-Aviv University, Ramat Aviv, Israel

Received for publication August 22, 2002. Accepted for publication February 4, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This study was supported by a grant from the Volkswagen-Stiftung Foundation (to M.L.) and a Minerva Postdoctoral Fellowship (to M.B.)

[2] Y.G. and M.B. contributed equally to this paper.

[3] Address correspondence and reprint requests to Dr. Mia Levite, Department of Neurobiology, The Weizmann Institute of Science, 76100 Rehovot, Israel. E-mail address: mia.levite@weizmann.ac.il

[4] Abbreviations used in this paper: MS, multiple sclerosis; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; EAE, experimental autoimmune encephalomyelitis; ECM, extracellular matrix; GluR3, glutamate receptor of AMPA subtype 3; MBP, myelin basic protein; NBQX, 6-nitro-7-sulfamoylbenzo[*f*]quinoxalin-2,3-dione; RE, Rasmussen's encephalitis; SDF-1α, stromal cell-derived factor 1α; TTX, tetrodotoxin; PicTX, picrotoxin; FN, fibronectin, LR, laminin receptor; GnRH, gonadotrophin-releasing hormone; KA, kainate; BCECF-AM, 2′,7′-bis(2-carboxy-ethyl)-5(6)-carboxyfluorescein acetoxy methyl ester; CXCL-12, CXC chemokine ligand-12.

 0022-1767/03/$02.00

divided into three subfamilies named after the glutamatergic agonist that causes their specific activation: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate (KA), and *N*-methyl-D-aspartate (NMDA). In each of these families, there are several subtypes identified by a number. There is currently no direct evidence for the presence of specific ionotropic or metabotropic receptors for glutamate on normal human T cells, despite reports on several types of glutamate receptors and/or transporters on a variety of other peripheral cells and tissues (15). Likewise, glutamate by itself has not been shown to activate T cell function.

In this study, we asked two questions: do T cells express ionotropic-receptor of the AMPA subtype 3 (GluR3); and can glutamate by itself trigger T cell function?

As to the first question, we focused specifically on GluR3 because it is not only a main synaptic receptor for glutamate but also is the source of autoantigen against which, in some human epilepsies, the immune system raises deleterious autoantibodies. These anti-GluR3 autoantibodies are suspected to play a role in epilepsy on the following basis: 1) some GluR3-immunized rabbits developed seizures (16); 2) some anti-GluR3 Abs, alike deleterious excess glutamate, can overactivate neurons (17), kill neurons and glia cells (18–20), and cause brain pathology (16, 21); 3) in humans, the presence of anti-GluR3 Abs is significantly associated with seizure frequency in particular types of epilepsy (22). Until now, the GluR3 autoantigenic receptor was shown to be expressed only in the nervous system, i.e., on neurons and glia cells.

We found, for the first time, that T cells from normal human individuals, an alloprimed human T cell clone, a human T leukemia line (Jurkat), and a mouse anti-myelin basic protein (MBP) T cell line express high levels of GluR3, identical in sequence with brain GluR3. We further found that glutamate by itself (in the absence of additional stimulatory molecules) and by direct interaction with its AMPA receptors triggers a key T cell function, the integrin-mediated adhesion to laminin and fibronectin (FN). Normally, T cells adhere to these major ECM glycoproteins only when the cells are activated. Glutamate by itself also markedly up-regulated the chemotactic migration of T cells toward the stromal cell-derived-factor-1α chemokine (SDF-1α), also termed CXC chemokine ligand-12 (CXCL12). This important chemokine is constitutively expressed both in the periphery and in the nervous system and plays a key role in numerous immune and neuronal functions.

Materials and Methods

Materials

L-Glutamate, KA, and PMA were from Sigma-Aldrich (St. Louis, MO); AMPA, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 6-nitro-7-sulfamoylbenzo[*f*]quinoxalin-2,3-dione (NBQX), tetrodotoxin (TTX), and picrotoxin (PicTX) were from Tocris Cookson (Bristol, U.K.); total human brain RNA was from Clontech Laboratories (Palo Alto, CA); and GluR3B and GluR3A peptides were synthesized at the Weizmann Institute of Science.

Sources of Abs and sera were: rabbit polyclonal anti-GluR2/3 C-terminal intracellular peptide (Chemicon International); mouse anti-67kDa laminin receptor (LR) mAb (LR Ab-1, clone MluC5; NeoMarkers, Fremont, CA); mouse anti-human CD29, VLA-5, and VLA-6 mAbs (Serotec, Oxford, U.K.); FITC-conjugated anti-rat, anti-rabbit and anti-mouse Abs (Jackson ImmunoResearch Laboratories, West Grove, PA), PE-conjugated mouse anti-human TCRαβ mAb (Serotec and BD PharMingen, San Diego, CA); PE-conjugated hamster anti-mouse TCRαβ mAb (BD PharMingen); mouse anti-human CXCR4 mAb (R&D Systems, Minneapolis, MN); normal rabbit, mouse, and goat serum (Jackson ImmunoResearch Laboratories).

Human T cells

Normal peripheral human T cells were purified from the fresh peripheral blood samples of healthy donors as described (11). The resulting cell population consisted of >95% T cells, as evaluated by TCR staining and flow cytometry, using a FACS.

Human T cell clone

The CD4+ alloprimed human T helper clone was kindly provided by R. Wank, Ludwig-Maximilians-University (Munich, Germany), and maintained in culture as described (23).

Mouse anti-MBP$_{87-99}$ T cell line

The anti-MBP$_{87-99}$ Th cell line, derived from lymph nodes of female SJL/J mice, was established, propagated in culture, and tested for its specificity as previously described (11).

RT-PCR and DNA sequencing

Total RNA from T cells was prepared according to Tri Reagent (Molecular Research Center, Cincinnati, OH) protocol. First-strand cDNA was synthesized from 4 μg of total RNA in a final incubation volume of 40 μl, by using the Reverse Transcription System (Promega, Madison, WI). PCR was conducted in a 50-μl reaction mixture containing either 400 ng cDNA for all the T cell types or 50 ng for total brain cDNA for GluR3 amplification, 5 μl of 10× Optibuffer (Bioline, London, U.K.), 3 μl of 50 mM $MgCl_2$, 2.5 μl of 10 mM dNTP mix (Promega), 4 U of Bio-X-act DNA polymerase (Bioline) and 2.5 μl of each GluR3 primer (10 pmol/μl) or 2.5 μl of the S14 primers (0.67 pmol/μl). For S14 amplification, 50 ng cDNA were used.

The sequence of the primers (5′-3′) and the lengths of the product were as follows: GluR3 primer pair 1, upstream primer (GluR3 E4), CGATACTTGATTGACTGCGA; downstream primer (GluR3 E9), TAC TATGGTCCGATTCTCTG, 632 bp; GluR3 primer pair 2, upstream primer (GluR3 E3), GACGCAGATGTGCAGTTTGTCATC; downstream primer (GluR3 E6), TAGTGGTGCATTCTTGGCTTCAGG, 516bp. S14: upstream primer, GTCCATGTCACTGATCTTTCTGGC; downstream primer, GTTTGATGTGTAGGGCGGTGATAC, 166 bp.

Conditions for PCR were 94°C for 1 min, 60°C for 40 s, and 72°C for 40 s (29 cycles for S14 PCR and 38 cycles for GluR3 PCR). The cDNA sequencing was performed with an automated sequencer at the sequencing unit of the Weizmann Institute of Science to confirm the identity of the PCR products.

Production and purification of anti-GluR3B Abs

Lewis rats were injected in both hind footpads with 100 μl (50 μl/foot) emulsion of mineral oil containing 200 μg of the GluR3B peptide (NEYERFVPFSDQQISNDSSSSENR, aa 372–395) and 200 μg of *Mycobacterium tuberculosis* (CFA; Difco, Detroit, MI). After 4–6 wk, the rats received a booster i.p. injection of 100 μg of peptide in PBS. Rats immunized with GluR3A peptide (NNENPMVQQFIQRWVRLDEREF PEAKNAP, aa 245–274; data not shown) or with PBS alone served as controls. The IgG from the immune and normal rat sera was purified on protein G columns using γ-Bind Plus Sepharose (Pharmacia, Knowhill, Milton Keynes, U.K.) according to the manufacturer's protocol.

Fluorescence immunocytochemical analysis

Normal peripheral human T cells were pelleted (1500 × *g*, 10 min, 4°C), suspended in 4% paraformaldahyde (1 × 10^6 cells/ml, 10 min, 22°C), centrifuged (10 min, 1500 × *g*), resuspended (1 × 10^6 cells/ml 80% ethanol), and pipeted onto gelatin-coated glass slides. The cells were then dried (2 h, 4°C), washed (5 min, three times with PBS), permeabilized (3 min with 0.5% Triton X-100), and incubated in a blocking medium (10% normal goat serum, 2% BSA, 1% glycine, 0.5% Triton X-100). The cells were then exposed to the rat polyclonal anti-GluR3B IgG Ab or to normal rat IgG for control (3.4 mg/ml, 12 h, 4°C) or to a rabbit polyclonal anti-GluR2/3 Ab or normal rabbit serum for control (2 μg/ml, 12 h, 4°C). After incubation, the cells were exposed to the appropriate FITC-conjugated secondary Abs (1/100 dilution, 2 h, 22°C). Fluorescence was visualized by fluorescence microscopy using a green filter.

Immunofluorescence staining and flow cytometry analysis for GluR3

Normal peripheral human T cells (isolated from fresh human blood samples), T leukemia line (Jurkat) and a mouse T cell line alloreactive to MBP$_{87-99}$ were subjected to double immunofluorescence staining, using our newly produced rat polyclonal anti-GluR3B IgG Ab (25 μg/ml/1 × 10^6cells/100-μl tube; 30 min on ice), or normal rat IgG for control. The cells were then stained with FITC-conjugated goat anti-rat IgG (100 μl of 1/100 dilution) and PE-conjugated mouse anti-human (for the normal and

Jurkat human T cells) or hamster anti-mouse (for the mouse anti-MBP 87–99 T cells) anti-TCR$\alpha\beta$ mAb (2 μl of stock). Cells stained with the second and third Abs only served as additional negative controls. Fluorescence profiles were recorded in a FACS.

Immunofluorescence staining and flow cytometry for CXCR4

Normal human T cells isolated from fresh PBL were subjected to double-immunofluorescence staining, using a mouse monoclonal anti-CXCR4 IgG Ab (10 μg/ml/1 $\times$ 10^6cells/100-μl tube; 30 min on ice) or normal mouse serum for control. The cells were then stained with an FITC-conjugated goat anti-mouse IgG (100 μl of 1/100 dilution) and PE-conjugated mouse anti-human TCR$\alpha\beta$ mAb (20 μl of stock). Cells stained with the second and third Abs only served as additional negative controls. Fluorescence profiles were recorded in a FACS.

T cell extraction and immunoblotting

T cells from healthy donors, a mouse (SJL/J) anti-MPB$_{87-99}$ line, or a human cortical neuronal cell line (HCN) (24) were suspended in PBS, washed by centrifugation (twice at 4000 rpm for 1 min), resuspended in buffer A (25) (composed of: 50 mM β-glycerophosphate (pH 7.3), 1.5 mM EGTA, 1.0 mM EDTA, 1.0 mM DTT, and 0.1 mM deaerated sodium), centrifuged again, resuspended in buffer H (25) (composed of: 0.1 mM β-glycerophosphate (pH 7.3), 1.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 mM sodium vanadate, 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 μg/ml pepstatin-A), and then disrupted on ice by sonication (three times for 7 s each with 20-s rest intervals). Cell homogenates were centrifuged (5 min at 15,000 rpm), and the supernatants were collected, subjected to protein determination, resolved by polyacrylamide gel electrophoresis (10% SDS-PAGE), and transferred to a nitrocellulose membrane. After transfer, blots were blocked (PBS plus 0.05% Tween plus 5% milk), washed extensively, and hybridized with Abs (1 μg/ml) against GluR3 (either the rat anti-GluR3B IgG or the polyclonal anti-GluR3/2 Ab; Chemicon). After extensive washing, blots were incubated with an appropriate anti-rat or anti-rabbit HRP-conjugated secondary Ab. The targeted proteins were visualized by ECL.

T cell adhesion to FN and laminin

Microtiter plates were covered with either FN (1 μg/ml; Sigma) or laminin (1 μg/ml; ICN Biomedicals, Aurora, OH), and the adhesion of normal resting human T cells that either remained untreated or were incubated with glutamate, AMPA, KA, or PMA (positive control) was assayed as described (11).

Blocking glutamate/AMPA-induced T cell adhesion to laminin by specific antagonists

Purified normal human T cells were suspended in adhesion medium (RPMI 1640 supplemented with 0.1% BSA) and pretreated with CNQX or NBQX (0.1 μM), the glutamate/AMPA receptor antagonists, or with the nonrelevant ion channel blockers TTX (1 μM) or PicTX (10 μM). After 5 min, the cells were exposed (30 min, 37°C, 7.5% CO_2 humidified incubator) to glutamate or AMPA (10 nM). The cells were then seeded in the laminin-coated microtiter plates, and the adhesion was monitored as described (11).

Involvement of specific integrins in glutamate-induced T cell adhesion to laminin

Freshly purified human T cells were treated (30 min, 37°C) with mAbs (15–25 μg/ml) specific to the human integrins (CD29, and α_5, α_6 chains of the VLA integrins) or with anti-67-kDa nonintegrin LR mAb (1/50 dilution). The T cells were then treated with glutamate (10 nM) and incubated (30 min, 37°C, 7.5% CO_2 humidified incubator). The treated cells were seeded in laminin-coated microtiter plates and returned to the incubator for an additional incubation (30 min), and the adhesion was monitored as described (11).

In vitro chemotactic migration assay

Normal human T cells were pretreated with glutamate or AMPA (10 nM, 18 h, 37°C, 7.5% CO_2 humidified incubator), and their chemotactic migration toward CXCL12/SDF-1α (1–250 ng/ml) was determined by FACS (fixed counting time, 2.0 min/sample) as described (12, 26).

In each experiment, the counting of the experimental samples by FACS (12, 26) started only after initial verification that once set for 2 min counting, the FACS analyses equal volumes of medium sucked from a series of control samples.

Statistical analysis

Statistical significance was analyzed by Student's t test.

Results

Human peripheral T cells, T leukemia cell line, and a T cell clone express the mRNA encoding the ionotropic glutamate receptor GluR3

To investigate the possible expression of GluR3 in T cells, we amplified cDNA from human peripheral T cells purified from fresh blood samples, an alloprimed human T cell clone (23), the Jurkat human leukemia T cell line, and total human brain (for positive control) by quantitative RT-PCR, using two sets of specific primers for GluR3. Parallel RT-PCR for the ribosomal protein S14 was conducted for normalization. Fig. 1*A* shows that all types of T cells tested express the specific GluR3 mRNA. The GluR3 RT-PCR amplification products in T cells are of the expected molecular mass for each set of primers respectively (E4–E9, 632 bp and E3–E6 516 bp), and identical with the RT-PCR GluR3 products of human brain (the possibility that the GluR3 PCR products were amplified from contaminating genomic DNA rather than from cDNA was excluded because amplification of the genomic GluR3 DNA, spanning the introns, would result in much larger PCR products). All types of T cells also harbored, as expected, the control S14 transcript (Fig. 1*A*, *bottom*).

To further confirm the GluR3 mRNA expression, we isolated the GluR3 cDNA fragments and subjected them to sequence analysis. The T cell RT-PCR amplification products using the two sets of GluR3-specific primers had sequences identical with that encoding the brain GluR3 protein (Fig. 1*B*).

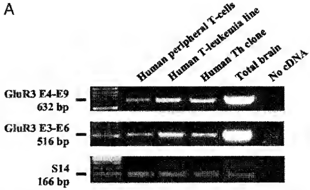
Production of specific anti-GluR3 Abs

To examine the expression of GluR3 at the protein level, we produced specific Abs to the GluR3B peptide (aa 372–395) derived from the extracellular domain of the receptor. This peptide is an autoantigen for anti-GluR3 Abs, found in some epileptic patients (17, 18, 27). To obtain specific anti-GluR3 Abs, which may serve for detection of this receptor, rats were immunized with the GluR3B peptide, and the anti-GluR3B IgGs were purified from the serum on protein G columns. The binding specificity of the purified anti-GluR3B IgG preparation was determined by ELISA, using microtiter plates coated with either GluR3B or GluR3A peptide (aa 245–274, another unique antigenic peptide of GluR3) or with a nonrelevant control peptide derived from herpes simplex DNA polymerase. The results showed strong and specific binding of the anti-GluR3B IgGs to the GluR3B peptide (Fig. 2*A*, black bar), but not to the GluR3A peptide or the control herpes simplex peptide (Fig. 2*A*). On the basis of this specific binding profile, the rat anti-GluR3B IgGs were used for future experiments.

The GluR3 protein is expressed in T cells

To study GluR3 at the protein level, human T cells were stained with two different anti-GluR3 Abs: the polyclonal rat anti-GluR3B IgG described above; and a commercial rabbit polyclonal Ab directed against a C-terminal intracellular peptide which is nearly identical in GluR3 and GluR2. Upon examination by fluorescence microscopy techniques, immunoreactivity toward both the extracellular and the intracellular peptides was observed (Fig. 2*B*). Whereas the rat anti-extracellular N-terminal GluR3B peptide Abs produced mainly a membranal staining pattern (Fig. 3*B*, *g–h*), the anti-intracellular C-terminal GluR3/2 peptide Abs stained the T cell cytoplasm (Fig. 3*Bj*). The GluR3 staining was specific, because T cells exposed to control Abs purified from rats injected

A



B

^{447}**gacgcagatgtgcagtttgtcatc**cagatgcgcccagccttgaag E3
gcgctattctgagtcttctgggtcattacaagtgggagaagtttgtg
tacctctatgacacagaacgag] [gattttccatcctccaagcgatt
atggaagcagcagtgcaaaacaactggcaagtaacagcaaggtctgt
gggaaacataaaggacgtccaagaattcaggcgcatcattgaagaaa
tggacaggaggcaggaaaag^{699}**cgatacttgattgactgcga**agtcg E4
aaaggattaacacaattttggaacag] [gttgtgatcctagggaaac
actcaagaggttatcactacatgctcgctaacctg] [ggttttactg
atattttactggaaagagtcatgcatgggggagccaacattacaggt
ttccagattgtcaacaatgaaaaccctatggttcagcagttcataca
gcgctgggtgaggctggatgaaagggaattc**cctgaagccaagaatg** E6
caccacta^{963}aag] [tatacatctgcattgacacacgacgcaatact
ggtcatagcagaagctttccgctacctgaggaggcagcgagtagatg
tgtcccggagaggaagtgctggagactgcttagcaaatcctgctgtg
ccctggagtcaaggaattgatattgagagagctctgaaaatg] [gtg
caagtacaaggaatgactggaaatattcaatttgacacttatggacg
taggacaaattataccatcgatgtgtatgaaatgaaagtcagtggct
ctcgaaaa] [gctggctactggaacgagtatgaaaggtttgtgcctt
tctcagatcagcaaatcagcaatgacagtgcatcct**cagagaatcgg** E9
accatagta^{1331}

FIGURE 1. Expression of GluR3 mRNA in different human T cell types. *A*, cDNA from human peripheral T cells, human T cell leukemia line Jurkat, and a human Th clone were amplified by semiquantitative RT-PCR. Total human brain cDNA served as a positive control. RT-PCR was performed using the GluR3 E4-E9 (*upper panel*), and E3–E6 (*middle panel*) specific primer pairs (shown in *B*). The lower panel represents the RT-PCR product obtained using ribosomal S14 primers, which served for normalization. The results indicate that all three T cell types express GluR3 mRNA. *B*, cDNA sequence of the GluR3 region amplified by RT-PCR in normal human peripheral T cells. The underlined nucleotides indicate the location of the PCR primers GluR3 E3, E4, E6, and E9 ("E" stands for the corresponding exons in the genomic DNA). The breakages (][) represent the locations in which there are introns in the genomic DNA. The amplified cDNA sequence of the T cell is identical with the known cDNA sequence of neuronal GluR3 mRNA (GenBank accession no. NM 007325).

with PBS (Fig. 2*Bf*) or to normal rabbit serum (Fig. 2*B*, *i*) did not stain.

Finally, the presence of the GluR3 protein in normal human T cells was confirmed by Western blot, using the rat anti-GluR3B IgG. A major immunoreactive band of the expected ~108,000-kDa mass (28) was detected in the normal human T cells and in human cortical neuronal cells (HCN) (24) serving as positive control (Fig. 2*C*).

Human peripheral T cells, T leukemia line and mouse Ag-specific T cell line express cell surface GluR3

To demonstrate the cell surface expression of the GluR3 protein on T cells, immunofluorescence staining and flow cytometry analysis were performed. Staining T cell suspensions with either rat anti-GluR3B purified polyclonal IgG or isotype control rat IgG and then with FITC-conjugated goat anti-rat IgG Ab and PE-conjugated anti-human TCR$\alpha\beta$ mAb (to confirm the T cell origin of the cells) showed that most of the purified normal peripheral human T cells express both GluR3 and TCR (framed windows of Fig. 3*Ab*, showing 74.3% specific double-positive staining, and Fig. 3*Aa*, showing 12.8% isotype control nonspecific staining). Histogram analysis further demonstrated the high expression of GluR3 on TCR$\alpha\beta^{+}$ cells (Fig. 3*Ac*, the solid bold line representing specific staining with rat anti-GluR3 Ab, compared with the broken line representing staining with the isotype control Ab).

In freshly isolated T cells from different human donors, the amount of GluR3 expression varied within a range of 30–85%. Variations are often observed in various T cell features and functions between T cell populations originating from different individuals (8, 12).

To further verify the cell surface expression of GluR3, we used a commercial polyclonal Ab raised against a GluR3 N-terminal peptide (thus far untested for its suitability for FACS). The results showed ~27% specific staining for GluR3 and CD3 double-positive cells, compared with ~9% of nonspecific staining with the isotype control Ab or with no first Ab (data not shown).

To explore the possible relevance of glutamate receptor expression to T cell cancers (i.e., T cell leukemia and T cell lymphoma), we tested whether GluR3 is expressed on the surface of human leukemic Jurkat T cells. We found 93.6% of the leukemic Jurkat T cells to be GluR3- and TCR-positive, compared with 17.6% of the cells stained nonspecifically with an isotype control IgG (Fig. 3*Bb* and *a*, respectively). These results clearly indicate that Jurkat malignant T leukemia cells express GluR3. High GluR3 staining on the TCR$\alpha\beta^{+}$ Jurkat T cells is also evident from the histogram analysis (Fig. 3*Bc*).

We further asked whether glutamate/AMPA receptors of the GluR3 subtype are expressed also on MS-associated autoimmune T cells. We postulated that during MS, direct stimulation of glutamate receptors on T cells by glutamate released from neurons within the CNS could be of importance, because it may contribute to the constitutive or recurrent activation of autoaggressive T cells, which attack and destroy the nerve-enwrapping myelin sheath. Interestingly, several studies reported recently that the treatment of mice (29) or rats (30) suffering from experimental autoimmune encephalomyelitis (EAE) (the animal model for MS), with NBQX, the specific AMPA/KA receptor antagonist, resulted in a substantial amelioration of disease. Because the presence on T cells of specific glutamate/AMPA receptors was not suspected, the beneficial effects of the AMPAR antagonists were attributed solely to the block of glutamate/AMPA receptors expressed on neurons and glia cells.

To test whether EAE-associated encephalitogenic T cells express glutamate/AMPA receptors, we used a mouse T cell line (9) specifically directed against MBP_{87-99} peptide, one of the key autoantigens in MS. We found clear indication for the expression of GluR3 on a significant proportion of TCR^{+} anti-MBP_{87-99} T cells (Fig. 3*Cb* and *a* framed windows, showing 41.5% specific staining compared with 9.4% of nonspecific staining with an isotype control Ab). Interestingly, a proportion of the anti-MBP_{87-99} T cells was TCR^{+} and GluR3^{-}.

To confirm the GluR3 expression on the anti-MBP_{87-99} T cells, we performed a Western blot analysis using both our purified rat anti-GluR3B IgG and a commercial affinity purified anti-GluR3/2 polyclonal Ab, which recognizes a nearly identical C-terminal sequence in GluR2 and GluR3. This polyclonal Ab produces in Western blots two poorly separated bands corresponding to GluR3 and GluR2 (GluR2 migrates slightly ahead of GluR3), without any cross-reacting with other GluR subtypes. The results show that the

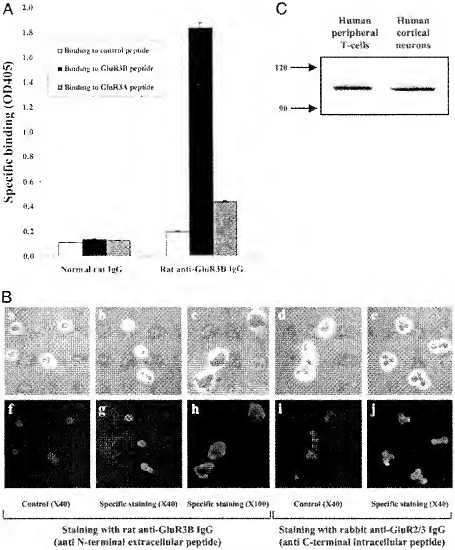


FIGURE 2. Fluorescence microscopy staining and Western blot of GluR3 in human peripheral T cells. *A*, Specific binding profile of purified newly produced rat anti-GluR3 IgG. Rat Abs to a GluR3 extracellular peptide (GluR3B), originating from GluR3B-immunized rats were purified on protein G columns and tested by ELISA for their binding to GluR3B peptide (■), GluR3A peptide (▨), or control peptide derived from herpes simplex DNA polymerase (□). Control IgG purified from sera of normal nonimmunized rats showed negligible binding to all three peptides. The results represent the mean ± SD of duplicate wells (1/1000 dilution) from one of two experiments performed. *B*, Fluorescence microscopy staining of GluR3 in human peripheral T cells. T cells were fixed on glass slides and stained with purified rat anti-GluR3B IgG, the GluR3B located at the N-terminal extracellular domain of the receptor (*b* and *c*, phase contrast; *g* and *h*, green filter) or normal rat IgG as control (*a*, phase contrast; *f*, green filter), followed by FITC-conjugated anti-rat IgG Ab. A membranal staining pattern is observed (×40 and ×100 magnifications). Cells were also stained with a commercial rabbit polyclonal Ab, directed against a C-terminal intracellular peptide which is nearly identical in GluR3 and GluR2 (*e*, phase contrast; *j*, green filter) or with normal rabbit serum as control (*d*, phase contrast; *i*, green filter), followed by FITC-conjugated anti-rabbit IgG. A cytoplasmic-like staining pattern is observed (×40 magnification). One representative experiment of three is shown. *C*, Immunoblotting of GluR3 in normal human T cells and in human cortical neuronal cells (24), using the rat anti-GluR3B IgG, yielded a band at the expected molecular mass of ~108,000 kDa. Similar results were obtained using T cells from five different individuals. Control blot did not yield any band.

two types of Abs detected a GluR3-specific immunoreactive band of the expected size on the mouse anti-MBP_{87-99} T cells (Fig. 3*Cc*).

Taken together, the above results show a GluR3 expression on the surface of the vast majority of normal human peripheral T cells, cancerous (leukemia)-related human T cells, and autoimmune (MS)-related mouse T cells.

Glutamate and AMPAR agonists cause, and specific antagonist prevent, T cells adhesion to laminin and FN

Regulated adhesion to the ECM is a key immune function playing a critical role in numerous physiological and pathological settings. It is essential for the transmigration of leukocytes through the blood vessels and their subsequent migration into resting tissues in general and inflamed tissues in particular (31). Recent studies have shown that the integrin-mediated adhesion to laminin also plays a critical role during EAE, as the encephalitogenic T cells transmigrate across the blood-brain barrier into the CNS, via laminin-containing endothelial cell membranes (32). Binding to major components of the ECM such as laminin takes place via specific adhesion receptors, either members of the integrin family or non-integrin molecules (12). It is widely accepted that only when specific integrins are activated can such adhesion to ECM glycoproteins take place, whereas resting leukocytes show very low basal adhesion.

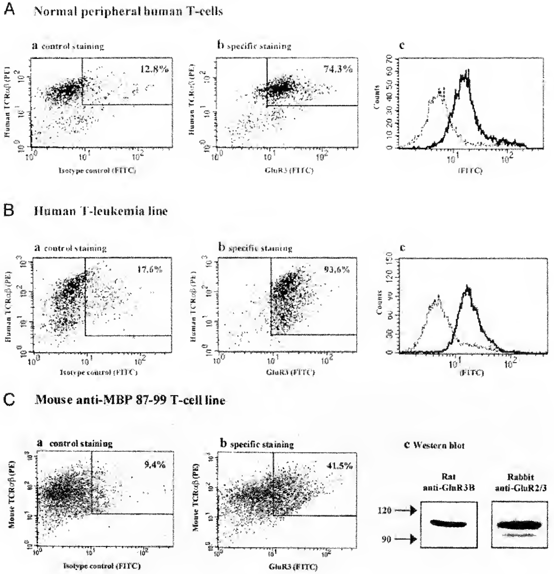


FIGURE 3. GluR3 is expressed on the surface of human peripheral T cells, a human T leukemia cell line, and a mouse anti-MBP T cell line. *A*, Freshly isolated normal human T cells were subjected to double immunofluorescence staining using rat anti-GluR3B IgG, FITC-conjugated anti-rat IgG (second Ab), and PE-conjugated anti-human TCR$\alpha\beta$ mAb (third Ab) (*b* and solid line in *c*), or using isotype control normal rat IgG and similar second and third Abs (*a* and broken line in *c*). A double-positive cell population for both GluR3 and TCR is clearly detected (framed window in *b*). The fluorescence intensity histograms in *c* represent single staining for GluR3 (solid line) or isotype control (broken line), of the TCR$\alpha\beta^+$ population. One representative experiment of eight is shown. *B*, Human T cell leukemia line (Jurkat) subjected to double-immunofluorescent staining using either the rat anti-GluR3B IgG (*b* and solid line in *c*) or rat isotype control (*a* and broken line in *c*), FITC-conjugated anti-rat IgG and PE-conjugated anti-human TCR$\alpha\beta$ mAb. The fluorescence intensity histograms in *c* represent single staining for GluR3 (solid line) or isotype control (broken line), of the TCR$\alpha\beta^+$ population. *C*, Anti-MBP$_{87-99}$ Th cell line was subjected to double-immunofluorescent staining using either the purified rat anti-GluR3B IgG (*a*) or isotype control Abs (*b*), FITC-conjugated anti-rat IgG and PE-conjugated anti-mouse TCR$\alpha\beta$ Ab. One representative experiment of seven is shown. Immunoblotting of GluR3 in mouse anti-MBP$_{87-99}$ T cell line, using the rat anti-GluR3B IgG (*left lane*) and a rabbit anti-GluR3/2 C-terminal polyclonal Ab (*right lane*) is also shown (*c*).

To determine whether glutamate by itself can activate the T cell integrins and endow the cells with an ability to adhere to laminin, we treated normal human peripheral T cells with 0.1 mM–0.01 pM glutamate (in the absence of any additional stimulating molecules) and assayed their adhesion to laminin-coated microtiter plates. The results showed that glutamate, at the micromolar to picomolar range, can cause T cell adhesion to laminin (Fig. 4*A*). The effective range of glutamate concentrations is in line with our previous observations on the direct effects of other neurotransmitters on T cell function (8, 9, 11).

To examine whether glutamate-induced T cell adhesion is mediated by specific AMPA/glutamate receptors, we stimulated T cells with either glutamate or two of the AMPAR agonists AMPA and kainate (Table I, all in 10 nM) and assayed their adhesion to laminin- or FN-coated microtiter plates. Nonspecific T cell activation with PMA served as a positive control. The results showed that both glutamate and the specific AMPA receptor agonists endowed T cells with the ability to adhere to laminin (Fig. 4*B*) and FN (Fig. 4*C*). The extent of adhesion induced by glutamate and its receptor antagonists was comparable with that induced by the potent phorbol ester PMA. We further tested whether CNQX and NBQX (Table I), two highly selective AMPAR antagonists, block the effects of glutamate and AMPA. These antagonists were used in a concentration range reported to be effective for the inhibition

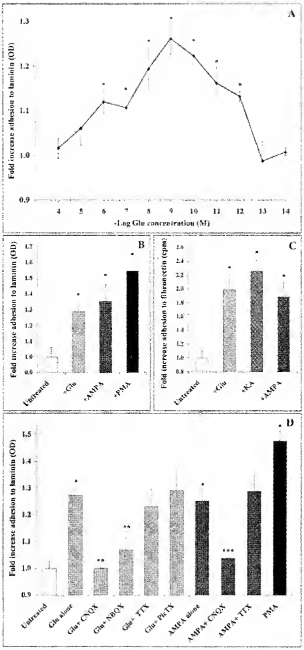


FIGURE 4. Glutamate (Glu) and AMPA receptor agonists induce adhesion of human T cells to laminin and FN via AMPARs. *A*, Glutamate causes the adhesion of T cells to laminin in a dose-dependent manner. Normal human T cells were treated with 10^{-14}-10^{-4} M glutamate and tested for their adhesion to laminin as described. The results are presented as means of four independent experiments, and expressed as fold increase ± SEM of the number of adhering cells (*, $p < 0.05$ vs untreated cells). *B* and *C*, Normal human T cells purified from blood samples of different human donors were pretreated (30 min, 37°C) with glutamate, AMPA, KA (10 nM), or PMA and tested for their adhesion to laminin (*B*) or FN (*C*). The mean fold increase ± SEM of adhesion to laminin or FN from four independent experiments using T cells from four individuals is shown. (*, $p < 0.05$ vs untreated). *D*, T cell adhesion to laminin caused by 10 nM concentrations of either glutamate or AMPA is blocked by the specific AMPAR antagonists CNQX or NBQX (both at 0.1 μM) but not by the nonrelevant ion channel blockers TTX (1 μM) and PicTX (10 μM). The results are presented as mean fold increase ± SEM of the number of cells that adhered to laminin. In repeated experiments using T cells from different human donors, each blocker was examined at least twice for its blocking effect. *, $p < 0.05$ vs untreated; **, $p < 0.05$ vs glutamate alone; ***, $p < 0.05$ vs AMPA alone.

Table I. *Agonists, antagonists, blockers, and anti-integrin Abs used in the study*

Effector	Function	Target
AMPA	Agonist	AMPAR
Kainate	Agonist	Kainate/AMPAR
CNQX	Antagonist	AMPAR
NBQX	Antagonist	AMPAR
TTX	Antagonist	Na^+ channel
PicTX	Antagonist	GABAR[a]
Anti-CD29 mAb	Blocker	β_1 integrin chain (binds FN and laminin)
anti-VLA-5 mAb	Blocker	α_5 integrin chain (binds FN)
Anti-VLA-6 mAb	Blocker	α_6 integrin chain (binds laminin)
Anti-67-kDa LR mAb	Blocker?	67-kDa nonintegrin LR

[a] GABAR, γ-aminobutyric acid receptor.

of neuronal AMPA-evoked responses. Two nonrelevant blockers served for control: TTX, a Na^+ channel blocker, and PicTX, a γ-aminobutyric acid receptor antagonist (Table I). The results presented in Fig. 4*D* demonstrate that the activating effects of both glutamate and AMPA are selectively blocked by CNQX and NBQX, but not by any of the control blockers. Taken together, these results show for the first time that glutamate can directly activate a T cell function and that it induces T cell adhesion to ECM components via the stimulation of specific AMPARs.

The glutamate-induced T cell adhesion to laminin is mediated by the $\alpha_6\beta_1$ integrins

To show that glutamate causes T cell adhesion to laminin by up-regulating the function of the specific $\alpha_6\beta_1$ laminin-binding integrins of the T cell, we used mAbs specific to these integrin moieties and control Abs directed against nonrelevant integrin moieties (Table I). The results presented in Fig. 5 show that the effect of glutamate was specifically blocked by anti-VLA-6 (anti-α_6 integrin chain), and anti-CD29 (anti-β_1 chain) mAbs. In contrast, no blocking effect was exerted by the nonrelevant anti-VLA-5 mAb (anti-α_5 integrin chain, which does not bind laminin) and by the anti-67-kDa nonintegrin LR mAb. These results demonstrate that glutamate-induced T cell adhesion to laminin is mediated by specific recognition and binding of $\alpha_6\beta_1$ integrins to laminin.

Glutamate increases the in vitro chemotactic migration of T cells

Alike adhesion to laminin and FN, the migration of T cells toward a chemokine (chemotaxis) is a key immune event crucial in numerous physiological and pathological conditions. It enables T cells to migrate and extravasate in a directional and regulated manner from the blood stream into chemokine-containing tissues. On these grounds, we investigated whether glutamate can up-regulate the chemotaxis of human T cells toward the potent and vital chemokine CXCL12/SDF-1. This chemokine and its specific receptor, the CXC chemokine receptor 4 (CXCR4), are crucial for chemotaxis of leukocyte subsets and endothelial cells (33) and for hemopoiesis, and are key players in a variety of additional immune functions. CXCL12/SDF-1 is constitutively expressed in bone marrow, heart, liver, kidney, and most importantly in the brain (34), where it is abundantly expressed and affects various neuronal and glial functions, including neurotransmission, neuronal migration, and plasticity. Accordingly, it was even suggested that CXCL12/SDF-1 is as essential to the nervous system as it is to the immune system (35).

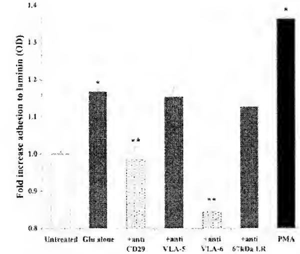


FIGURE 5. Glutamate-induced adhesion of T cells to laminin takes place via binding of laminin by the $\alpha_6\beta_1$ integrins. Normal human T cells were pretreated with mAbs directed against different human integrin moieties or against the 67-kDa nonintegrin laminin receptor (Table I), exposed to glutamate (10 nM, 30 min, 37°C) and then tested for their ability to adhere to laminin-coated wells. The results are expressed as the mean fold increase ± SEM of the number of cells that adhered to laminin. One experiment of two performed is presented. *, $p < 0.05$ vs untreated; **, $p < 0.05$ vs glutamate alone.

To examine whether glutamate causes T cells to migrate toward CXCL12/SDF-1, we used the Boyden chemotaxis chamber method (36) and counted the fluorescence-labeled normal human T cells that migrated through a laminin-coated filter from an upper chamber containing medium to a lower chamber containing CXCL12/SDF-1.

First, we confirmed that indeed the presence of the chemokine in the lower chamber was essential for the chemotactic migration of normal untreated resting human T cells (Fig. 6*A*). We then observed that T cells treated with glutamate (10 nM) migrated to a much larger extent toward CXCL12/SDF-1, in comparison with untreated cells (Fig. 6*B*). Thus, glutamate significantly increased the chemotactic migration of normal human T cells toward CXCL12/SDF-1. The glutamate-specific receptor agonist AMPA also augmented the chemotactic migration of T cells toward CXCL12/SDF-1 (Fig. 6*B*), suggesting that the prochemotactic effects of glutamate and AMPA were mediated specifically by AMPARs. Dose-response experiments showed that the extent of the glutamate-mediated chemotactic migration depended on the concentration of the CXCL12/SDF-1 chemokine, with a threshold at a concentration ≥10 ng/ml (Fig. 6*C*).

The glutamate-induced increase in the T cell chemotaxis toward CXCL12/SDF-1 was specifically mediated by CXCR4, the highly specific membranal receptor for this chemokine, given that anti-CXCR4 mAb fully blocked it (Fig. 6*D*).

Finally, glutamate-induced augmented chemotaxis toward CXCL12/SDF-1 was not accompanied by an increased CXCR4 expression, because immunofluorescence staining with anti-CXCR4 mAb showed a similarly high level of TCR^+CXCR4^+ expression in the untreated and glutamate-treated T cells (Fig. 6*E*, upper vs lower fluorescence profiles). Alternative mechanisms that may account for glutamate-induced effects are discussed below.

Discussion

The physiological factors able to directly activate T cell function in vivo in nonlymphoid tissues such as the brain, and allowing a direct communication between T cells and components of the CNS environment, are still unknown. Their discovery may have important scientific and clinical implications.

In recent years, we found that several neurotransmitters and neuropeptides, among them dopamine, somatostatin, substance P, calcitonin-gene-related peptide, neuropeptide Y, and GnRH I and II are able on their own and at physiological concentrations to stimulate their receptors expressed on the T cell surface and trigger various T cell functions (8, 9, 11, 37). Among these functions are de novo gene expression (12), cytokine secretion (9), integrin-mediated adhesion (8), in vitro chemotactic migration, and in vivo homing to specific organs (12). On this basis, we asked here whether glutamate, the major CNS excitatory neurotransmitter, could also by itself trigger T cell function.

The present study provides for the first time the evidence, based on the combination of specific GluR3 RT-PCR, sequencing, immunohistofluorescence staining, Western blot, and flow cytometry, that normal peripheral human T cells, cloned alloprimed human T cells, cultured human T leukemia cells, and mouse autoimmune-associated anti-MBP_{87-99} T cells express high levels of ionotropic glutamate receptors of the AMPA subtype 3. Our sequencing data further show an identity between the T cell-expressed GluR3 and the brain GluR3. We have not conducted yet the electrophysiological recordings needed to determine whether the T cell GluR3 receptor channel has properties similar to those of the neuronal GluR3. We also do not know yet whether the GluR3 channels in T cells are coupled to the same signaling pathways as in neurons and glia cells and whether their opening causes a Ca^+ influx (important, e.g., for the neuronal death induced by excess glutamate). These future investigations may provide an explanation for our interesting observation (data not shown) that, in contrast to neurons (13), exposure of T cells to excess glutamate (0.1–10 mM) did not cause excitotoxic T cell death (as assessed by cell viability measurements of various T cell types based on trypan blue exclusion). Whatever is the explanation or mechanism, we speculate that such property may enable T cells to survive and function in the CNS in the pathological conditions associated with excess glutamate, known to massively kill neurons and glia cells. Although we focused on GluR3, further studies are required to investigate whether T cells express additional members of the ionotropic glutamate receptor family. In addition, it is likely that human T cells express metabotropic (G-coupled) glutamate receptors, because such receptors have been identified on mouse thymocytes and thymic stromal cell lines (38). T cells are not the first example of peripheral cells expressing glutamate receptors, because several types of glutamate receptors have been reported on a variety of other peripheral cells and tissues (15).

Glutamate itself, in the absence of any additional molecules, is found here for the first time to directly cause the adhesion of T cells to two principal ECM glycoproteins, laminin and FN, and to trigger thereby the activation of a key T cell function which takes place only when the respective integrin moieties are activated. The glutamate-induced T cell adhesion is mediated via specific glutamate receptors of the AMPA subtype expressed on T cells, given that it is mimicked by two highly specific AMPAR agonists and blocked by the respective antagonists.

Interestingly, the glutamate dose response shows that T cells cannot be prompted to integrin-mediated adhesion at very high glutamate concentrations ($>1 \times 10^{-5}$ M). Because the concentration of glutamate in the plasma is ~30–80 μmol ($3–10 \times 10^{-5}$ M) (e.g., 79.4 ± 41.8 μmol/L according to Ref. 39 and 32 ± 4 μmol/L according to Ref. 40), our observation suggests that frequent glutamate-T cell interactions in blood do not necessarily lead to constitutive (and perhaps undesired) integrin activation and to

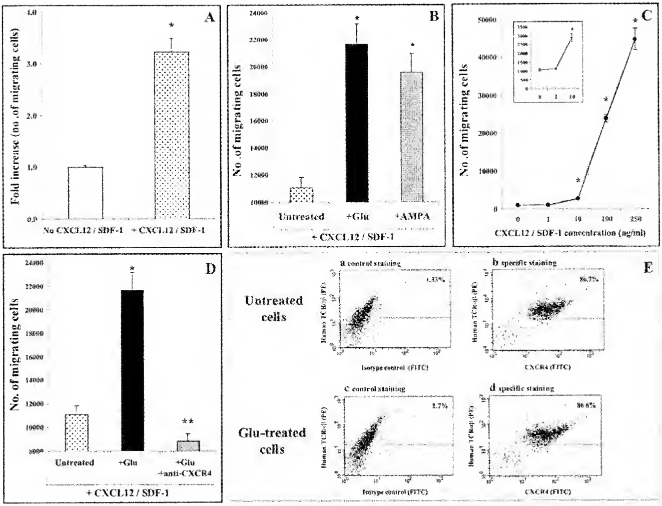


FIGURE 6. Glutamate (Glu) augments chemotactic migration of normal human T cells toward the chemokine CXCL12/SDF-1. *A*, Human T cells migrate toward a chemokine. Normal peripheral T cells purified from blood samples of human donors were labeled with the fluorescent dye 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxy methyl ester (BCECF-AM) and tested for their migration across laminin-coated filters toward either the chemokine CXCL12/SDF-1 (250 ng/ml) or control PBS. The results are expressed as the mean fold increase ± SEM in the number of T cells that migrated toward CXCL12/SDF-1, as compared with the number of T cells that migrated toward chemokine-free medium, in six independent experiments using T cells from six individuals. *B*, Glutamate and AMPA up-regulate the extent of T cell chemotactic migration. Human T cells purified from fresh blood samples were pretreated (18 h, 37°C) with 10 nM glutamate or AMPA, labeled with the fluorescent dye BCECF-AM, and tested for their migration toward CXCL12/SDF-1. The number of fluorescently labeled cells that transmigrated through the laminin-coated filters from the upper chamber to the chemokine-containing lower chamber was evaluated by flow cytometry (*, $p < 0.05$ vs untreated). One experiment of four, using T cells from four different individuals, is shown. *C*, Dose response for glutamate-induced T cell chemotactic migration. Human T cells pretreated (18 h, 37°C) with 10 nM glutamate, and loaded with BCECF-AM were tested for their migratory capacity toward 0, 1, 10, 100, and 250 ng/ml CXCL12/SDF-1 (in the lower chamber). One experiment of two is presented. *, $p < 0.05$ vs no chemokine. *D*, The chemotaxis of glutamate-treated normal human T cells was mediated by CXCR4, because anti-CXCR4 mAb fully blocked the glutamate-induced augmented chemotaxis toward CXCL12/SDF-1. One experiment of three performed is presented. *, $p < 0.05$ vs untreated; **, $p < 0.05$ vs glutamate. *E*, The augmented chemotactic migration induced by glutamate was not due to an increased expression of CXCR4, the membranal receptor for CXCL12/SDF-1, as immunofluorescence staining with anti-CXCR4 mAb showed a similarly high level of expression of TCR^+CXCR4^+ in untreated (upper) and glutamate-treated (lower) cells. One representative experiment of three is shown.

the subsequent T cell adhesion to laminin and FN. However, because glutamate concentration in the CSF is lower than in the plasma, the estimated concentration being ~3–4 μM (0.3–04 × 10^{-5} M) (41), we speculate that T cell encounters with glutamate in the brain are more likely to result in the activation of the T cell integrins and a subsequent cellular adhesion and migration.

In this study, we further observe that glutamate increases the migration of normal human T cells toward the potent chemokine CXCL12/SDF-1, which is constitutively expressed in the periphery and in the nervous system (34). Interestingly, a recent study reveals the existence of a physical association between CXCR4 and the AMPAR subtype 1 (GluR1) in cerebellar granule neurons (42). If CXCR4 is also physically associated with AMPAs in T cells (an issue currently under investigation), this could perhaps account for the observed glutamate-induced chemotaxis of T cells to CXCL12/SDF-1. Because the glutamate-induced augmented chemotaxis was not accompanied by an increased CXCR4 expression, further studies are required to unveil the mechanism responsible for this effect. Indeed, several studies have suggested that other factors besides the CXCR4 membranal expression level regulate its function: Baribaud et al. (43) found that CXCR4 exists in T cells in multiple conformational states and proposed that these have functional consequences on chemokine receptor function; whereas Nguyen et al. (44) raised the idea that lipid rafts may play a regulatory role in CXCL12/SDF-1 signaling and that membranal cholesterol may modulate receptor conformation and subsequent

binding of CXCL12/SDF-1. Moreover, sialylated O-glycans and sulfated tyrosines may contribute to the high affinity binding of CXCR4 to CXCL12/SDF-1, as recently shown for the CCR5 chemokine receptor which also plays an important role in leukocyte chemotaxis and activation (45). Regardless of the explanation or mechanism, our observations suggest that in certain contexts CXCL12/SDF-1 and glutamate may act in concert for a common cause, the recruitment of T cells to specific sites, in the nervous and immune systems.

As to further indications that glutamate can modulate immune functions, Lombardi et al. (46) found recently that glutamate can significantly potentiate the in vitro activating effects of anti-CD3 mAb or PHA, suggesting that it is capable of modulating the proliferation and Ca^{2+} influxes triggered by certain other molecules. However, glutamate by itself, in a concentration range of 10 nM–1 mM, could not trigger either Ca^{2+} influxes or the proliferation of a heterogeneous population of human PBMN (consisting of B and T lymphocytes, monocytes, and polymorphonuclear leukocytes) (46).

We speculate that glutamate-induced T cell activation revealed herein may be either beneficial or detrimental depending on whether T cell reactivity is required (e.g., T cell-mediated clearance of encephalomyelitis-inducing virus from the brain, and T cell-mediated protective autoimmunity) (4–6). We further speculate that the specific expression of GluR3 on T cells and the ability of glutamate to trigger T cell function may be highly relevant to various physiological and pathological conditions, including the following exemplary instances.

Functional interactions between T cells and glutamate in the context of MS

MS and its animal model EAE are demyelinating diseases caused by autoreactive T cells, which attack the nerve-enwrapping myelin sheath (1, 2, 29, 30). It was recently found that during the course of EAE, adhesion to laminin in the CNS plays a crucial role in the recruitment, transmigration, and penetration of autoaggressive T cells: the parenchymal basement membranes containing certain laminin isoforms were permissive for encephalitogenic T cell transmigration; whereas those containing others were restrictive (32). On the basis of our findings reported herein, we suggest that encounter of T cells with glutamate could cause their adhesion to laminin-containing brain parenchyma and could further promote their directional migration toward chemokines secreted in specific sites within the CNS.

Our findings of the ability of glutamate by itself to activate T cells may be highly relevant to MS, also due to an additional set of important observations; treatment of mice (29) or rats (30) sensitized for EAE with NBQX, the AMPA/kainate antagonist, resulted in substantial amelioration of disease, increased oligodendrocyte survival, and reduced dephosphorylation of neurofilament H, an indicator of axonal damage (29). It was then concluded that NBQX was beneficial for EAE because it blocked glutamate/AMPA receptors expressed on neuronal or glia cells. Our present findings call for a reinterpretation of these results and suggest that in vivo NBQX suppressed EAE because on top of inhibiting glutamate receptors on neurons and glia, it also blocked the AMPA receptors expressed on the autoaggressive encephalitogenic T cells. We thus suggest that by blocking T cell expressed AMPA receptors, NBQX prevented the in vivo activation of the autoaggressive T cells by glutamate released from nerve endings at the sites of inflammation/damage in the CNS, thereby reducing their pathogenic potential and conferring EAE suppression.

Source of the GluR3-derived autoantigen GluR3B and relevance to epilepsy

Specific Abs to GluR3 have been suggested to contribute to the etiology and pathology of several forms of human epilepsies (16, 22). The primary autoantigen of the potentially pathogenic anti-GluR3 Abs, identified as the GluR3B peptide, can be generated by the specific cleavage of the parent GluR3 by granzyme B, a serine protease released by activated immune cells (but only if an internal N-linked glycosylation sequon within the GluR3-granzyme B recognition sequence (ISND*S) is not glycosylated (27)). Until now, because GluR3 was known to be expressed only on neurons and glia cells, it was assumed that the anti-GluR3 Abs are raised only against the CNS GluR3. However, the finding in this study of GluR3 expression on T cells reveals a novel source of the GluR3 autoantigen and suggests that anti-GluR3 Abs may be also raised against a peripheral GluR3. We speculate that if so, the anti-peripheral GluR3 Abs, upon gaining access to the CNS, may encounter the brain GluR3 and interfere with neuronal and glial signaling and survival, thereby promoting neuropathology and epilepsy.

All the above suggestions, although requiring further investigations and validation, are illustrations of how the expression of GluR3 on T cells and the direct activation of T cell functions by glutamate could have profound scientific and clinical implications. Probably, only the tip of the iceberg has been uncovered thus far.

Acknowledgments

We thank Professor V. I. Teichberg for fruitful discussions and incisive comments on the manuscript. We also thank Professor Zelig Eshhar and Tova Wax for their guidance in purification of the anti-GluR3 IgGs, Professor R. Wank for a gift of the $CD4^+$ alloprimed human T helper clone, and Alon Chen for his help in the initial immunohistochemical staining.

References

1. Hemmer, B., S. Cepok, S. Nessler, and N. Sommer. 2002. Pathogenesis of multiple sclerosis: an update on immunology. *Curr. Opin. Neurol. 15:227.*
2. Merrill, J. E., and E. N. Benveniste. 1996. Cytokines in inflammatory brain lesions: helpful and harmful. *Trends Neurosci. 19:331.*
3. Tompkins, S. M., K. G. Fuller, and S. D. Miller. 2002. Theiler's virus-mediated autoimmunity: local presentation of CNS antigens and epitope spreading. *Ann. NY Acad. Sci. 958:26.*
4. Binder, G. K., and D. E. Griffin. 2001. Interferon-γ-mediated site-specific clearance of alphavirus from CNS neurons. *Science 293:303.*
5. Drescher, K. M., S. L. Johnston, W. Hogancamp, G. H. Nabozny, C. S. David, I. J. Rimm, P. J. Wettstein, and M. Rodriguez. 2000. $V\beta8^+$ T cells protect from demyelinating disease in a viral model of multiple sclerosis. *Int. Immunol. 12:271.*
6. Moalem, G., R. Leibowitz-Amit, E. Yoles, F. Mor, I. R. Cohen, and M. Schwartz. 1999. Autoimmune T cells protect neurons from secondary degeneration after central nervous system axotomy. *Nat. Med. 5:49.*
7. Shrikant, P., and E. N. Benveniste. 1996. The central nervous system as an immunocompetent organ: role of glial cells in antigen presentation. *J. Immunol. 157:1819.*
8. Levite, M., L. Cahalon, R. Hershkoviz, L. Steinman, and O. Lider. 1998. Neuropeptides, via specific receptors, regulate T cell adhesion to fibronectin. *J. Immunol. 160:993.*
9. Levite, M. 1998. Neuropeptides, by direct interaction with T cells, induce cytokine secretion and break the commitment to a distinct T helper phenotype. *Proc. Natl. Acad. Sci. USA 95:12544.*
10. Levite, M. 2001. Nervous immunity: neurotransmitters, extracellular K^+ and T cell function. *Trends Immunol. 22:2.*
11. Levite, M., Y. Chowers, Y. Ganor, M. Besser, R. Hershkovits, and L. Cahalon. 2001. Dopamine interacts directly with its D3 and D2 receptors on normal human T cells, and activates β_1 integrin function. *Eur. J. Immunol. 31:3504.*
12. Chen, A., Y. Ganor, S. Rahimipour, N. Ben-Aroya, Y. Koch, and M. Levite. 2002. The neuropeptides GnRH-II and GnRH-I are produced by human T cells and trigger laminin receptor gene expression, adhesion, chemotaxis and homing to specific organs. *Nat. Med. 8:1421.*
13. Choi, D. W. 1992. Excitotoxic cell death. *J. Neurobiol. 23:1261.*
14. Sattler, R., and M. Tymianski. 2001. Molecular mechanisms of glutamate receptor-mediated excitotoxic neuronal cell death. *Mol. Neurobiol. 24:107.*
15. Gill, S. S., and O. M. Pulido. 2001. Glutamate receptors in peripheral tissues: current knowledge, future research, and implications for toxicology. *Toxicol. Pathol. 29:208.*

16. Rogers, S. W., P. I. Andrews, L. C. Gahring, T. Whisenand, K. Cauley, B. Crain, T. E. Hughes, S. F. Heinemann, and J. O. McNamara. 1994. Autoantibodies to glutamate receptor GluR3 in Rasmussen's encephalitis. *Science 265:648.*
17. Twyman, R. E., L. C. Gahring, J. Spiess, and S. W. Rogers. 1995. Glutamate receptor antibodies activate a subset of receptors and reveal an agonist binding site. *Neuron 14:755.*
18. Levite, M., I. A. Fleidervish, A. Schwarz, D. Pelled, and A. H. Futerman. 1999. Autoantibodies to the glutamate receptor kill neurons via activation of the receptor ion channel. *J. Autoimmun. 13:61.*
19. Whitney, K. D., and J. O. McNamara. 2000. GluR3 autoantibodies destroy neural cells in a complement-dependent manner modulated by complement regulatory proteins. *J. Neurosci. 20:7307.*
20. Koustova, E., Y. Sei, L. Fossom, M. L. Wei, P. N. Usherwood, N. B. Keele, M. A. Rogawski, and A. S. Basile. 2001. LP-BM5 virus-infected mice produce activating autoantibodies to the AMPA receptor. *J. Clin. Invest. 107:737.*
21. Levite, M., and A. Hermelin. 1999. Autoimmunity to the glutamate receptor in mice: a model for Rasmussen's encephalitis? *J. Autoimmun. 13:73.*
22. Mantegazza, R., P. Bernasconi, F. Baggi, R. Spreafico, F. Ragona, C. Antozzi, G. Bernardi, and T. Granata. 2002. Antibodies against GluR3 peptides are not specific for Rasmussen's encephalitis but are also present in epilepsy patients with severe, early onset disease and intractable seizures. *J. Neuroimmunol. 131:179.*
23. Besser, M., and R. Wank. 1999. Cutting edge: clonally restricted production of the neurotrophins brain-derived neurotrophic factor and neurotrophin-3 mRNA by human immune cells and Th1/Th2-polarized expression of their receptors. *J. Immunol. 162:6303.*
24. Ronnett, G. V., L. D. Hester, J. S. Nye, K. Connors, and S. H. Snyder. 1990. Human cortical neuronal cell line: establishment from a patient with unilateral megalencephaly. *Science 248:603.*
25. Silverman, M. A., O. Benard, H. Jaaro, A. Rattner, Y. Citri, and R. Seger. 1999. CPG16, a novel protein serine/threonine kinase downstream of cAMP- dependent protein kinase. *J. Biol. Chem. 274:2631.*
26. Petit, I., M. Szyper-Kravitz, A. Nagler, M. Lahav, A. Peled, L. Habler, T. Ponomaryov, R. S. Taichman, F. Arenzana-Seisdedos, et al. 2002. G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. *Nat. Immunol. 3:687.*
27. Gahring, L., N. G. Carlson, E. L. Meyer, and S. W. Rogers. 2001. Granzyme B proteolysis of a neuronal glutamate receptor generates an autoantigen and is modulated by glycosylation. *J. Immunol. 166:1433.*
28. Hampson, D. R., X. P. Huang, M. D. Oberdorfer, J. W. Goh, A. Auyeung, and R. J. Wenthold. 1992. Localization of AMPA receptors in the hippocampus and cerebellum of the rat using an anti-receptor monoclonal antibody. *Neuroscience 50:11.*
29. Pitt, D., P. Werner, and C. S. Raine. 2000. Glutamate excitotoxicity in a model of multiple sclerosis. *Nat. Med. 6:67.*
30. Smith, T., A. Groom, B. Zhu, and L. Turski. 2000. Autoimmune encephalomyelitis ameliorated by AMPA antagonists. *Nat. Med. 6:62.*
31. von Andrian, U. H., and C. R. Mackay. 2000. T cell function and migration. two sides of the same coin. *N. Engl. J. Med. 343:1020.*
32. Sixt, M., B. Engelhardt, F. Pausch, R. Hallmann, O. Wendler, and L. M. Sorokin. 2001. Endothelial cell laminin isoforms, laminins 8 and 10, play decisive roles in T cell recruitment across the blood-brain barrier in experimental autoimmune encephalomyelitis. *J. Cell Biol. 153:933.*
33. Kim, C. H., and H. E. Broxmeyer. 1999. Chemokines: signal lamps for trafficking of T and B cells for development and effector function. *J. Leukocyte Biol. 65:6.*
34. Pillarisetti, K., and S. K. Gupta. 2001. Cloning and relative expression analysis of rat stromal cell derived factor-1 (SDF-1): SDF-1α mRNA is selectively induced in rat model of myocardial infarction. *Inflammation 25:293.*
35. Tham, T. N., F. Lazarini, I. A. Franceschini, F. Lachapelle, A. Amara, and M. Dubois-Dalcq. 2001. Developmental pattern of expression of the α chemokine stromal cell-derived factor 1 in the rat central nervous system. *Eur. J. Neurosci. 13:845.*
36. Falk, W., R. H. Goodwin, Jr., and E. J. Leonard. 1980. A 48-well microchemotaxis assembly for rapid and accurate measurement of leukocyte migration. *J. Immunol. Methods 33:239.*
37. Levite, M. 2001. Nerve-driven immunity: the direct effects of neurotransmitters on T cell function. *Ann. NY Acad. Sci. 917:307.*
38. Storto, M., U. de Grazia, G. Battaglia, M. P. Felli, M. Maroder, A. Gulino, G. Ragona, F. Nicoletti, I. Screpanti, L. Frati, and A. Calogero. 2000. Expression of metabotropic glutamate receptors in murine thymocytes and thymic stromal cells. *J. Neuroimmunol. 109:112.*
39. Reynolds, J. D., D. W. Amory, H. P. Grocott, W. D. White, and M. F. Newman. 2002. Change in plasma glutamate concentration during cardiac surgery is a poor predictor of cognitive outcome. *J. Cardiothorac. Vasc. Anesth. 16:431.*
40. Divino Filho, J. C., S. J. Hazel, P. Furst, J. Bergstrom, and K. Hall. 1998. Glutamate concentration in plasma, erythrocyte and muscle in relation to plasma levels of insulin-like growth factor (IGF)-I, IGF binding protein-1 and insulin in patients on haemodialysis. *J. Endocrinol. 156:519.*
41. Danbolt, N. C. 2001. Glutamate uptake. *Prog. Neurobiol. 65:1.*
42. Limatola, C., S. Di Bartolomeo, F. Trettel, C. Lauro, M. T. Ciotti, D. Mercanti, L. Castellani, and F. Eusebi. 2003. Expression of AMPA-type glutamate receptors in HEK cells and cerebellar granule neurons impairs CXCL2-mediated chemotaxis. *J. Neuroimmunol. 134:61.*
43. Baribaud, F., T. G. Edwards, M. Sharron, A. Brelot, N. Heveker, K. Price, F. Mortari, M. Alizon, M. Tsang, and R. W. Doms. 2001. Antigenically distinct conformations of CXCR4. *J. Virol. 75:8957.*
44. Nguyen, D. H., and D. Taub. 2002. CXCR4 function requires membrane cholesterol: implications for HIV infection. *J. Immunol. 168:4121.*
45. Bannert, N., S. Craig, M. Farzan, D. Sogah, N. V. Santo, H. Choe, and J. Sodroski. 2001. Sialylated *O*-glycans and sulfated tyrosines in the NH2-terminal domain of CC chemokine receptor 5 contribute to high affinity binding of chemokines. *J. Exp. Med. 194:1661.*
46. Lombardi, G., C. Dianzani, G. Miglio, P. L. Canonico, and R. Fantozzi. 2001. Characterization of ionotropic glutamate receptors in human lymphocytes. *Br. J. Pharmacol. 133:936.*